ABSTRACT

Nucleotide vector composition containing such vector and vaccine for immunization against hepatitis

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Nucleotide vector comprising at least one gene or one complementary DNA coding for at least a portion of a virus, and a promoter providing for the expression of such gene in muscle cells. The gene may be the S gene of the hepatitis B virus.

A vaccine preparation containing said bare DNA is injected into the host previously treated with a substance capable of inducing a coagulating necrosis of the muscle fibres.

Figure 1

NUCLEOTIDE VECTOR, COMPOSITION CONTAINING SUCH VECTOR AND VACCINE FOR IMMUNIZATION AGAINST HEPATITIS

The present application relates to a vector for immunization against hepatitis.

It is also related to a composition containing this vector.

Immunization by injection of bare DNA into muscle tissues has been the object of several studies since the beginning of the 1990s.

Thus, ULMER et al. (Science, 259, 1745-1749, 1993) obtained protection against the Influenza virus by induction of the cytotoxic T lymphocytes through injection of a plasmid coding for the Influenza A nucleoprotein into the quadriceps of mice. The plasmid used carries either the Roùs sarcoma virus promoter or the cytomegalo virus promoter.

RAZ et al. (Proc. Natl. Acad. Sci. USA, 90, 4523-4527, 1993) injected vectors comprising the Rous sarcoma virus promoter and a gene coding for interleukin-2, interleukin-4 or the β1-type transforming growth factor (TGF-β1). The humoral and cell immune responses of the mice to which these plasmids have been intramuscularly administered are improved.

WANG et al. (Proc. Natl. Acad. Sci. USA, 90, 4156-4160, 1993) injected a plasmid carrying a gene coding for the envelope protein of the HIV-1 virus into mice muscles. The plasmid injection was preceded by

treatment with bupivacaine in the same area of the muscle. The authors demonstrate the presence of antibodies capable of neutralizing the HIV-1 virus infection. However, it will be noted that DNA was injected twice a week for a total of four injections.

DAVIS et al. (Compte-Rendu du 28ème Congrès Européen sur le muscle, Bielefeld, Germany, 21-25 September 1992) injected plasmids carrying a luciferase or β-galactosidase gene coding by pretreating the muscles with sucrose or a cardiotoxin. The authors observed the expression of luciferase or β-galactosidase.

More recently, an article published in Science et Avenir (September 1993, pages 22-25) indicates that WHALEN et DAVIS succeeded in immunizing mice against the hepatitis B virus by injecting pure DNA from the virus into their muscles. An initial injection of snake venom toxin, followed 5 to 10 days later by a DNA injection, is generally cited. It is specified that this is not a practical method.

These studies were preceded by other experiments in which various DNAs were injected, in particular into muscle tissues. Thus, the PCT/US application N° 90/01 515 (published under N° WO-90/11 092) discloses various plasmid constructions which can be injected in particular into muscle tissues for the treatment of muscular dystrophy. However, this document specifies that DNA is preferentially injected in liposomes.

This also applies to Canadian patent CA-362.966 (published under N° 1.169.793) which discloses the

intramuscular injection of liposomes containing DNA coding in particular for HBs and HBc antigens. The results described in this patent mention the HBs antigen expression. The presence of anti-HBs antibodies was not investigated.

International application PCT/FR 92/00 898 (published under N° WO-93/06 223) discloses viral vectors which can be conveyed to target cells by blood. These vectors are thus recognized by the cell receptors, such as the muscle cells, and can be used in the treatment of muscular dystrophy or of thrombosis.

This application does not relate to immunization against viruses such as, for example, that of hepatitis B.

Thus, it arises from the state of the art cited that although immunization techniques against hepatitis by injection of bare DNA are already known, these techniques had many disadvantages which made their implementation impractical.

Furthermore, the bare DNA used to vaccinate the mice was pure DNA from the virus. This type of treatment can not be considered for human vaccination due to the risks involved for the patients.

Finally, the earliest experiments in which the injected DNA is contained in liposomes did not demonstrate any immune response.

The applicant has therefore aimed at discovering new vector constructions allowing immunization against hepatitis without having a detrimental effect on human health.

He has further aimed at finding an additive for compositions containing the constructions which would allow an effective degeneration of muscle tissue before the DNA injection, and compatible with the requirements of human health.

The applicant has surprisingly shown that it is possible to achieve an effective and durable level of antibodies much greater than the level permitting to obtain in man an efficient and durable immune protection against infection by the hepatitis virus, by administering by intramuscular injection a vector with defined construction, and a substance capable of inducing a coagulating necrosis of the muscle fibres.

The present application thus relates to a nucleotide vector comprising at least:

- a gene or a complementary DNA coding for at least a part of the virus protein, and

- a promoter allowing the expression of this gene in muscle cells.

Said vector may not replicate in these cells.

It may also be replicative, allowing to obtain a high number of copies per cell and to enhance the immune response.

The vector is also chosen in order to avoid its integration into the cell's DNA, such integrations being known to activate the oncogens and induce cell canceration.

The vector according to the present invention is advantageously a plasmid of partly bacterial origin and notably carrying a bacterial replication origin and a

gene allowing its selection, such as a gene for resistance to an antibiotic.

This vector may also be provided with a replication origin allowing it to replicate in the muscle cells of its host, such as the replication origin of the bovine papilloma virus.

The gene or the complementary DNA included in this vector advantageously codes for a structure protein of a virus but it can also code for a regulatory protein.

The gene or complementary DNA carried by this vector can code for a least a portion of a hepatitis virus protein, in particular hepatitis B, and preferentially the protein HBs, in one of its forms S, S-preS2 or S-preS2-preS1, in which case the gene is gene S.

The virus may also be responsible for another hepatitis such as a hepatitis A or of a non-A, non-B hepatitis, such as a hepatitis C, E or delta.

The gene or protein sequences for these hepatitis viruses are described or may be deduced from the following documents:

patent FR-79 21 811, patent FR 80.09.039, patent EP-81.400.634, patent FR 84.03.564, patent EP 91.830.479 and the article by Najarian et al. (Proc. Natl. Acad. Sci. USA, 1985, 82, 2627-2631).

The vector may also include genes coding for at least a portion of the gp160 protein of HIV-1 virus associated with the p25 protein, and/or the p55

protein, and/or the p18 protein or at least a gene coding for the Rev protein of HIV-1 virus.

The vector may also include instead of a virus protein, a protein from a pathogenic micro-organism such as a protein from the bacterium causing diphtheria, whooping cough, listeriosis, the tetanus toxin etc.

The promoter carried by this vector is advantageously the promoter for the cytomegalovirus (CMV). It may however be any other promoter which allows the efficient expression of the gene in the muscle cells.

It may thus be:

- an internal or endogenic promoter, that is a promoter of the virus from which the gene is taken; such a promoter may be completed by a regulatory element of the muscle or another tissue, in particular an activating element,

- a promoter from a gene of a cytoskeleton protein, in particular desmine as described by BOLMONT et al. (Journal of submicroscopic cytology and pathology, 1990, 22, 117-122) et ZHENLIN et al. (Gene, 1989, 78, 243-254).

- the promoter from the virus HBV surface genes.

Generally, the promoter may be heterologous to the host, that is not naturally found in the host, but it is advantageously homologous, while being originally active in a tissue other than the muscle tissue.

In addition to the promoter, the vector may include a terminal transcription sequence, situated downstream of the gene.

Such vector may be the pCMV/HBS or pRCCMV-HBS plasmid, having the SEQ ID N°1 sequence, filed under N°I-1370 with the Collection Nationale des Cultures des Micro-organismes de l'Institut Pasteur (CNCM) on 21 October 1993.

It may also be the pRSV/HBS plasmid filed under N°I-1371 with the CNCM on 21 October 1993.

This plasmid has a similar structure to pCMV/HBS but includes the Rous sarcoma virus (RSV) promoter instead of the cytomegalovirus (CMV) promoter.

Other plasmids may be:

- pCMVHB-S1.S2.S constructed by inserting the fragment Bgl II-Bgl II of the S gene, obtained from pCP10, into a pBlueScript vector modified to contain supplementary cloning sites in the "polylinker" portion. The fragment containing the S gene was then removed by KpnI-BssH II digestion then cloned into the corresponding sites of pcDNA 3 (In vitrogen, Rad Systems Europe Ltd, Abingdon UK) so as to obtain pCMVHB-S1.S2.S. This plasmid was filed under N°I-1411 with the CNCM.

- pCMVHB-S2.S obtained by eliminating the pre-S1 part of the HBS gene from pCMVHB-S1.S2.S by KpnI/MstI digestion, then by bonding the two extremities after treatment with S1 nuclease.

pCMVHB-S2.S was filed with the CNCM under N°I-1410.

- pHBV-S1.S2.S, filed with the CNCM under N°I-1409, was obtained by inserting the S gene Bgl II- Bgl II fragment, obtained from pCP10, into a pBlueScript vector modified to contain supplementary cloning sites in the "polylinker" portion.

- pBS-SKT-S1.S2.S codes for the three envelope proteins S, S-preS_1 and S-preS_1-preS_2 of the HBV virus.

The present invention further relates to nucleotide sequences comprising a promoter homologous to the host and another regulatory sequence for the expression of a gene or complementary DNA coding for one of the above mentioned proteins.

The present invention further relates to a vaccine or medicine containing at least one vector, or a nucleotide sequence, such as defined above.

It further relates to a composition capable of inducing a cytotoxic response comprised of at least one nucleotide sequence expressed in the muscle cells and including a promoter such as defined above.

It further relates to a non-lipid pharmaceutical composition for immunization against a viral infection such as a hepatitis including, on the one hand, at least a substance capable of inducing a coagulating necrosis of the muscle fibres and, on the other hand, a vector such as described above or including one of the nucleotide sequences, complete or partial, such as described above. By partial sequence is meant a sequence coding for at least 6 amino acids.

Said substance is advantageously bupivacaine.

Advantageously, said composition is characterized in that the vector is administered in the muscle of the individual to immunized, at least 5 days after the administration of the bupivacaine, and substantially in the same location.

Such prior administration of bupivacaine surprisingly allows to increase the effectiveness of the vector administration as well as the immunization of the individual.

Advantageously, the vector is administered ten days after administration of bupivacaine, and substantially in the same location of the individual's muscle.

The present composition may also contain additives which are compatible and pharmaceutically acceptable.

Such composition is preferentially administered by intramuscular injection. The injection can be carried out using a syringe designed for such use or using a liquid jet gun such as described by FURTH et al. (1992, Anal. Biochem. 205, 365-368).

The quantity of bupivacaine required to obtain sufficient degeneration of the muscle tissue, in order to achieve optimal immunization, is in the order of 0.10 mg to 10 mg per dose of injected composition.

The quantity of vector to be injected in order to achieve optimal immunization of the individual against a hepatitis varies according to the protein coded by the gene carried by the vector. As an indication, between 0.1 and 1000 μg of vectors are injected per individual.

The vectors may be obtained by methods known to those skilled in the art, in particular by synthesis or by genetic engineering methods.

Such methods are those described in particular in the technical manual :

Maniatis T. et al. 1982 - Molecular Cloning, A Laboratory Manual, Cold Spring Harbour - Ed. New York.

The present invention is illustrated by, without in any way being limited to, the following examples, in which:

Figure 1 is a schematic representation of pRC/CMV-HBs plasmid.

Figures 2A to 2D are schematic representations of pCMVHB-S, pCMVHB-S2.S., pCMVHB-S1.S2.S and pHBV-S1.S2.S plasmids, respectively.

Figures 3, 4 and 5 are schematic restriction maps for pCMVHB-S2.S, pCMVHB-S1.S2.S and pRSV-HBS plasmids, respectively.

Figure 6 illustrates the secretion of antigenic HBs particles (HBs Ag) in ng/ml (ordinates) as a function of the number of days (abscissa) for cells carrying the pCMVHB-S, pCMVHB-S1.S2.S, pHBV-S1.S2.S, pSVS or pCMVHB-S2.S plasmids.

Figures 7A and 7B illustrate the determination on some particles in Figure 6 of the presence of the $preS_1$ and $PreS_2$ antigens using respectively anti-$preS_1$ and anti-$preS_2$ antibodies. The formation of antibody-antigen complexes is shown by the optical density (ordinates), as a function of antigen concentration.

Figures 8A to 8D represent the anti-HBS responses (HBS Ab as ordinate, expressed as mUI/ml) and anti-preS2 (preS2 Ab as ordinate, expressed in O.D.) of mice vaccinated by pCMVHB-S (8A), pCMVHB-S2.S (8B), pCMVHB-S1.S2.S (8C) and pHBV-S1.S2.S (8D), respectively.

Figure 9 illustrates the antibody response, IgG and IgM immunoglobulins (titre as ordinates), of a mouse vaccinated by pCMVHB-S2.S as a function of the number of weeks (abscissa).

Figures 10A to 10C represent the anti-group and anti-subtype ay responses induced by DNA from pCMV-S (DNA) or from the HBS antigen (prot), respectively in mice B10 (10A), B10S (10B) and B10M (10C).

Figures 10D to 10F represent the antigroup responses induced by DNA from pCMV-S (DNA) or from the HBS antigens (prot), respectively in mice B10 (10D), B10S (10E) and B10M (10F).

Figure 11 represents a linear restriction map for the pBS-SKT-S1.S2.S plasmid.

EXAMPLE 1 :

Induction of antibodies against a hepatitis B surface antigen by sequential injection of bupivacaine and of a plasmid carrying a gene coding for the antigen

1) Materials and methods

1.1 Bupivacaine pretreatment

All experiments were made on the muscles of the anterior tibia (AT) of mice C57BL/6J aged between 5 to 7 weeks.

A single degeneration-regeneration cycle of the muscle fibres is induced in the muscles of the anterior tibia of non-anaesthetized mice, by intramuscular injection of 50µl marcaine (bupivacaine 0.5%, DMSO 1%) sold by Laboratoires Astra, France. The solution is injected using a tuberculosis syringe with a needle fitted into a polyethylene sleeve, in order to limit the penetration depth to 2mm.

As marcaine is an anesthetic, injections into the right and left legs were performed at 10 to 30 minute intervals to prevent an overdose.

1.2 DNA preparation

The plasmid used was constructed by cloning into a modified pBlueScript vector of the Xho I-Bgl II restriction fragment of the pCP10 plasmid which contains the gene coding for the HBS surface antigen and the non-translated sequences, both upstream and downstream, including the polyadenylation signal.

The S gene was then recovered by digestion using KpnI-BssHII enzymes and the fragment was cloned into the site of the pRC/CMV vector sold by In Vitrogen. The final plasmid construction was called pCMV-HBS and was filed under N°I-1370 with the CNCM.

This plasmid is represented schematically in Figure 1. The CMV promoter is situated between the 288 nucleotide which is the cleavage position of MluI and the 896 nucleotide, which is the cleavage position of KpnI. The DNA fragment including the structural gene of the HBs antigen structure was cloned between the 896 and 2852 nucleotides (position of BssH III).

The HBs gene spreads between the 911 (XhoI position) and 2768 nucleotides (Bgl II position) respectively.

The complete sequence for this plasmid is sequence SEQ ID N°1.

The purified plasmid DNA was prepared by standard methods then redissolved in PBS buffer and stored at -20°C until the injection was performed.

1.3 DNA injection

One to five days after the marcaine injection, DNA was injected into the same area, the mouse being anaesthetized using sodium pentobarbital (75mg/kg interperitonal path).

The DNA solution which contains 50µg of plasmid DNA and 50µl of PBS buffer was injected by a single intramuscular injection through the skin into the anterior tibia muscles undergoing regeneration.

The injections were performed bilaterally into the two legs of the mice, each animal thus receiving a total of 100µg of recombinant plasmid DNA. As for the marcaine injection, the DNA solution was injected using the tuberculosis syringe with the needle described previously.

A single intramuscular DNA injection was performed in each leg.

2. Results

The results obtained are summarized in Table I below.

They show very clearly that a DNA injection after treatment with marcaine allows a large number of seric

antibodies to be obtained against the hepatitis B surface antigen.

These results are surprising, from the analysis of the state of the art it was not inferred that a plasmid would allow the induction of anti-HBs antibodies which could be found in the serum and thus allow an effective vaccination.

The ease of application of the plasmid vaccination, and the fact that boosters would not be necessary, allows the consideration of a large scale vaccination.

EXAMPLE 2 :

Comparison of the efficiency of a plasmid injection in the presence and absence of lipids.

A dose of 10µg plasmid DNA from the SV40-luciferase vector available commercially ("pGL2-Control Vector" from Promega, reference E1 11) in 50µl of physiological solution was injected into the sucrose pretreated muscle following the method of David et al. (Hum. Gene Ther. 4:151-159 (1993)). The injected DNA is mixed earlier with lipids such as dioctadecylamidoglycyl spermine (DOGS) or the following mixtures : DOGS + spermidine, and DOGS + polyethyleneglycol (PEG). The luciferase activity was determined 5 days after the injection.

These results are shown in table II below.

They show that the presence of lipids (DOGS) very reduces significantly the efficiency of the plasmid injection with respect to a composition with no lipids (control).

EXAMPLE 3:

Comparison of the responses of mice and rabbits to plasmids carrying different promoters and envelope genes for the HBV virus.

Four plasmids were constructed allowing the expression of one, two or three envelope proteins for the HBV virus. In three of the constructions (pCMVBH-S, pCMVHB-S2.S, pCMVHB-S1.S2.S) the genes coding for the HBV virus envelope proteins are put under transcriptional control of the promoter of the CMV virus precursor genes (Figure 1, Figure 2A to 2C, Figures 3 and 4). The fourth plasmid (pHBV-S1.S2.S) uses the promoter for the HBV virus surface genes contained in the pre-S1 region of this virus (Cattaneo et al. (1983) Nature, 305, 336) (Figure 2D) as a transcriptional controlling element. In the four constructions, the polyadenylation signal used is contained in the HBV sequences present in 3' of the S gene.

1. In vitro control of the vector efficiency.

To control the efficiency of these vectors in vitro in eucaryote cells, mouse fibroblasts or myoblasts were transfected. A plasmid expressing the three envelope proteins under control of the SV40 promoter (pSVS) was used as a control (Michel et al. 1984, Proc. Natl. Acad. Sci. USA, 81, 7708-7712)). Figure 6 illustrates the secretion kinetics of the HBs particles in the culture supernatants. The low antigen levels produced by transfection of the pCMVHB-S1.S2.S vector are compatible with a large degree of synthesis

of the large envelope protein starting from the CMV promoter. This protein being myristilised in its amino terminal region, is retained in the endoplasmic reticulum (Ganem, (1991), Current Topics in Microbiology and Immunology, 168, 61-83). Retention in the cell of proteins carrying the pre-S1 determinants was confirmed by immunofluorescence.

The composition of the secreted particles was analyzed in an ELISA sandwich system using as capture antibodies a monoclonal mouse antibody specific to the pre-S1 (Figure 7A) or pre-S2 (Figure 7B) determinants as capture antibodies and rabbit anti-HBs polyclonal serum as second antibodies. These experiments show that the AgHBs particles produced starting from pCMVHB-S1.S2.S vector carry pre-S1 and Pre-S2 determinants showing the presence of the large and medium envelope proteins of the HBV virus. Particles secreted after the transfection of the pCMVHB-S2.S and pHBV-S1.S2.S vectors carry, in addition to the HBs determinants, pre-S2 determinants characteristic of the medium envelope proteins.

2) DNA inoculation

DNA purified on a Quiagen column was injected by an intramuscular path in a single injection of 100µg (50µg/leg) in the anterior tibia muscle of mice C57/BL6 (8 mice per group). Five days prior to the injection, the muscle was pretreated with cardiotoxin in order to induce degeneration followed by regeneration of the muscles cells thus favoring the DNA capture by these cells.

The DNA injection experiments were also carried out for rabbits. In this case, pCMVHB-S DNA was administered into normal muscle without degeneration, either by using an injection gun without needle called Biojector^{R}, or by conventional syringes fitted with needles.

3) Anti-Hbs responses for mice vaccinated with DNA

An anti-HBs antibody response is induced by a single injection of one or other of the four plasmids used.

The antibody response was analyzed using a commercial anti-HBs antibodies detection kit (Monolisa anti-HBs, Diagnostic Pasteur). Anti-preS2 antibodies are detected by an ELISA system using, on the solid phase, a peptide from the pre-S2 (AA 120-145) region on the solid phase corresponding to a B major epitope carried by this area (Neuarth et al., (1985), Nature, 315, 154).

Figures 8A to 8D illustrate the anti-HBs (HBs-Ab) response kinetics expressed in milli-international units/ml and the anti-pre-S2 response (pres2Ab) determined as optical density (492nm) for 1/100 diluted serums. Detection was carried out using a mouse anti-immunoglobulin antibody (IgG) coded with peroxidase.

The injection of the pCMVHB-S plasmid (Figure 8A) induces a constant anti-HBs antibody synthesis. Seroconversion was observed in 100% of mice from one week after the injection with an antibody average level of 48mUI/ml (from 12 to 84 mUI/ml, standard deviation (SD) =28), which is 4 to 5 times superior to the

threshold required in man to provide protection (10 mUI/ml).

The induced response for a single injection of pCMVHB-S2.S plasmid (Figure 8B) is characterized by the very early apparition of anti-HBs antibodies. These antibodies reach an average level of 439 mUI/ml (from 104 to 835 mUI/ml; SD = 227) at one week then reduce before increasing again to reach the initial level at 13 weeks. The significance of this antibody peak will be discussed later. A peak for anti-pre-S2 IgG antibodies is observed at two weeks.

The appearance of anti-HBs antibodies induced by injection of pCMVHBV-S1.S2.S plasmids (Figure 8C) and pHBV-S1.S2.S (Figure 8D) is slightly delayed as the mice only seroconvert to 100% after two weeks. The seroconversion profile is identical, it is characterized by an initial response which is specific to the pre-S2 antigen followed by an anti-HBs response which gradually increases to reach a level of 488 mUI/ml (from 91 to 1034 mUI/ml; SD=552) (pCMVHB-S1.S2.S) and 1725 mUI/ml (from 143 to 6037 mUI/ml; SD=1808) (pHBV-S1.S2.S) at 13 weeks.

4) Anti-HBS response of rabbits injected with DNA

Results presented in tables III and IV show that the antibody levels detected at 8 weeks in rabbits immunized using the Biojector are significantly higher than those obtained by a DNA injection by needle.

5) Qualitative analysis of the humoral response

ELISA systems applied to the solid phase of the HBs antigens of varying composition with respect to the

determinants presented on the solid phase and using mouse antibodies specific to IgM or IgG as second antibodies gave a qualitative analysis of the antibody response that was achieved.

In all cases, the single injection of DNA in mice is characterized by the early appearance of AgHBs specific IgM followed immediately by conversion to IgG isotype antibodies which is characteristic of the memory response induced by the auxiliary T cells. The antibody response to the DNA injection is characterized by its prematurity. Indeed, seroconversion is achieved 8 to 15 days after the injection depending on the DNA type used and in all cases the plateau is achieved in four weeks and maintained constantly over a period of 12 weeks.

The use of the heterologous sub-type HBs antigens (ad) fixed on ELISA plates allows the formation/detection of the presence, in the serum of immunized mice, of antibodies specific to the anti-a group, and by difference in reactivity with respect to AgHBs of the same sub-type (ay), of antibodies specific to the anti-y sub-type. The presence of antibodies specific to determinants of the AgHBs group is very important as the former are capable of giving protection against the heterologous sub-type virus during virulent tests in chimpanzees (Szmuness et al. (1982) N. Engl. J. Med. 307, 1481-1486).

Analysis of the response induced by the pCMV-S2.S vector shows that it has a remarkable similarity with the one which can be observed in man during infection.

It is characterized by an extremely early (8 days) peak for IgM which is specific to the pre-S2 region immediately followed by conversion to anti-pre-S2 IgG (Figure 9). This response is followed by the appearance of IgM then IgG anti-HBs antibodies. The anti-HBs antibody production is constant and reaches a maximum after 4 weeks. At 13 weeks IgG anti-HBS and anti-pre-S2 remain at a constant level.

The anti-sub-group (y) response precedes that of the anti-group response (a) in the same way as that described for the vaccination with the recombinant vaccine (Tron et al., (J. Infect. Dis.160, 199-204).

The response obtained with the three other DNA vaccines illustrates the commutation of class IgM ——> IgG which is characteristic of the secondary response. The response being first of all directed against the sub-type before being against the AgHBs group determinants.

The long term response which was studied for pCMVHB-S DNA shows that the antibody peak is reached within 3 months and this remains at a constant level 6 months later (Table V).

6. Genetic vaccine and non-response

The high number of non-responders to the classical vaccine (2.5 to 5%) remains a major problem for vaccination against hepatitis B. It has been possible to correlate the non-response in man to certain HLA types (Krustall et al., (1992) J. Exp. Med. 175, 495-502) and to a defect in the antigen presentation or stimulation of the auxiliary T cells.

To study the possible impact of the genetic vaccination on the AgHBs non-response, a range of mice strains were used for which the response to various HBV virus envelope proteins is controlled genetically and has been well characterized by Millich et al. (1986 J. Immunol. 137, 315). The pCMVHB-S construction previously described was injected into B10 (H-2^b) B10.S (H-2^s) and B10.M (H-2^f) mice muscles.

The B10 strain responds to the three virus envelope proteins, the B10.S strain does not respond to AgHBs but this non-response can be overcome by immunization with HBsAg antigens which are carrying pre-S2 determinants. The B10M strain is totally non-responsive to both HBs and pre-S2 antigens. A response for the latter strain can be achieved by immunization using AgHBs carrying pre-S1 determinants.

The mice immunized by the DNA received a single injection (100μg) in the regenerating muscle. Control mice were injected with two intraperitonal injections of protein at an interval of one month, the first of 2μg AgHBs to which the complete Freund additive (CFA) was added and the second of 2μg AgHBs to which the incomplete Freund additive (IFA) was added.

The results obtained for pCMVHB-S are illustrated by Figures 10A to 10F.

- In the B10 strain (good responder) the DNA induced response is earlier than that induced by the protein after a single injection.

- The appearance of anti-HBs antibodies sub-type specific then group specific after immunization with

pCMVHB-S DNA was observed in the B10S strain (non-responder to AgHBS in the absence of pre-S2). Group specific anti-HBs antibodies are observed in HBs protein immunized mice only after the second injection.

- A group and sub-type specific anti-HBs response is obtained for DNA immunization of strain B10M (non-responder to AgHBs in absence of pre-S1) whereas only a sub-type specific response is induced by the protein with two injections being required.

The response induced by the three vector types is compared in the three mice strains.

CONCLUSIONS

It is generally thought that the humoral response to HBs antigens is sufficient by itself to give protection. The presence of antibodies directed against other determinants (pre-S1 and pre-S2) carried by the virus envelope proteins, themselves protectors, could improve the response quality. The experiments reported here as a whole illustrates that the humoral response induced by the genetic anti-hepatitis B vaccination is greater in several fields than that which can be achieved for the classical vaccination.

In terms of seroconversion levels: the 100% level is obtained, after only one injection, from day 8 for mice immunized with pCMV-HBS DNA and pCMVHB-S2.S.

In terms of response level: the 10 mUI/ml threshold level, considered sufficient to give protection in man, is always greatly exceeded.

In terms of the speed of response: in 8 days a very high level of anti-pre-S2 antibodies is obtained

for the pCMVHB-S2.S vector and it is known that the former are capable of giving protection by themselves (Itoh et al., (1986) Proc. Natl. Acad. Sci. USA 83, 9174-9178).

In terms of response stability: anti-HBs antibodies remain constant at a high level for more than 6 months.

In terms of response quality: type IgG antibodies characteristic of a response which is dependent on the auxiliary T cells and therefore on a memory response are obtained.

In terms of anti-viral activity: the antibodies are specific to the viral sub-type but especially group specific and therefore susceptible to giving a cross protection.

In terms of biological significance: the response profile obtained by pCMVHB-S2.S immunization mimes totally that which is observed in man after a resolved viral infection.

TABLE I

Induction of antibodies against the hepatitis B surface antigen

Description	Number of mice	Level of antibodies against hepatitis B surface antigen in the serum (mIU/ml)		
		Before DNA injection	15 days after DNA injection	35 days after DNA injection
DNA injected 1 day after marcaine treatment	5	0	average: 56 from 5 to > 140	average: 59
DNA injected 5 days after marcaine treatment	5	0	average: 71 from 21 to > 108	average: 47

TABLE II

Group	Luciferase RLU/sec/muscle (Average ± SEM) RLU = Relative Light Unit	Percentage relative to the control
Control	43 082 ± 5 419	100 %
4X DOGS	28 ± 7	0.06 %
DOGS - Spermidine	50 ± 23	0.12 %
PEG-DOGS	0 ± 0	0.00%

TABLE III

Immunization with the Biojector^{R}

pCMV-HB.S			
N°	0 weeks	2 weeks	8 weeks
2.1	0	517	380
2.2	0	374	322
3.1	0	258	418
4.1	0	400	4045
4.2	0	88	86
4.3	0	314	420
6.1	0	415	1001
6.2	0	1543	3517
6.3	0	1181	141
Average	0	566 mUI/ml	1148 mUI/ml
SD	0	476	1521
SEM	0	159	507
N	9	9	9
CV		84%	133%

TABLE IV

Immunization by injection using a needle

pCMV-HB.S			
N°	0 weeks	2 weeks	8 weeks
1.1	1	0	1
5.1	0	287	186
5.2	0	162	798
5.3	0	305	203
7.1	0	86	175
7.2	0	1108	dead
Average	0	325 mUI/ml	273 mUI/ml
SD	0	401	305
SEM	0	164	136
N	6	6	5
CV	245%	124%	112%

TABLE V

Long term response of a mouse vaccinated with pCMVHB-S

	1 month	2 months	3 months	6 months
* a-HBs titre in mUI/ml	227	662	1299	1082
* a-HBs ELISA titre	3.5×10^{-4}	5×10^{-4}	8.5×10^{-4}	9×10^{-4}

SEQUENCES LIST

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: INSTITUT PASTEUR

(B) STREET: 28, rue du Docteur Roux

(C) TOWN: PARIS

(D) COUNTRY: FRANCE

(E) POST CODE: 75724

(ii) TITLE OF THE INVENTION: Nucleotide vector, composition containing such vector and vaccine for immunization against hepatitis

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORMAT:

(A) SUPPORT TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) COMPUTER SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (OEB)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 5618 base pairs

(B) TYPE: nucleic acid

(C) NUMBER OF STRANDS: double

(D) CONFIGURATION: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGIN:

(B) STRAIN: pRCCMV-HBS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT 40

CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT 80

CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG 120

CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA 160

CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG 200

CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT 240

GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC 280
ATTAGTTCAT AGCCCATATA TGGAGTTCCG CGTTACATAA 320
CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC 360
CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT 400
AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC 440
TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT 480
ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG 520
TAAATGGCCC GCCTGGCATT ATGCCCAGTA CATGACCTTA 560
TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA 600
TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA 640
TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT 680
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AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC 760
CCCATTGACG CAAATGGGCG GTAGGCGTGT ACGGTGGGAG 800
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CTGCTTAACT GGCTTATCGA AATTAATACG ACTCACTATA 880
GGGAGACCCA AGCTTGGTAC CGGGCCCCCC CTCGAGGATT 920
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TCTTGAGTCC CTTTTTACCG CTGTTACCAA TTTTCTTTTG 1600
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GAAAATGGCC GCTTTTCTGG ATTCATCGAC TGTGGCCGGC 2960
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AGCGCATCGC CTTCTATCGC CTTCTTGACG AGTTCTTCTG 3120
AGCGGGACTC TGGGGTTCGA AATGACCGAC CAAGCGACGC 3160
CCAACCTGCC ATCACGAGAT TTCGATTCCA CCGCCGCCTT 3200
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GAGAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT 3480
GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC 3520
CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG 3560
AGCTAACTCA CATTAATTGC GTTGCGCTCA CTGCCCGCTT 3600
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TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG 5040
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GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA 5560
GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA 5600
AAAGTGCCAC CTGACGTC 5618

CLAIMS

1. Nucleotide vector comprising at least :

- a gene or complementary DNA coding for at least a portion of a virus protein, and

- a promoter allowing the expression of the gene in the muscle cells.

2. Vector according to claim 1, characterized in that the virus is that of a hepatitis.

3. Vector according to claim 1, characterized in that it does not replicate in the cells.

4. Vector according to claim 1, characterized in that the gene codes for at least a portion of the hepatitis B virus protein.

5. Vector according to claim 4, characterized in that the protein is the S, S-preS_2 or S-preS_2-PreS_1 protein.

6. Vector according to claim 4, characterized in that the gene is the S gene.

7. Vector according to claim 1, characterized in that the virus is that of hepatitis A or a non-A, non-B hepatitis, such as hepatitis C, E or delta.

8. Vector according to claim 1, characterized in that the promoter is the cytomegalovirus promoter.

9. Vector according to claim 1, characterized in that it is the pCMV-HBS plasmid filed under N° I-1370 with the CNCM on 21 October 1993.

10. Vector according to claim 1, characterized in that it is the pCMV-HBS-S1.S2.S plasmid filed with the CNCM under N° I-1411.

11. Vector according to claim 1, characterized in that it is the pCMVHB-S2.S plasmid filed with the CNCM under N° I-1410.

12. Vector according to claim 1, characterized in that it is the pRSV-HBS plasmid filed under N° I-1371 with the CNCM on 21 October 1993.

13. Vector according to claim 7, characterized in that the promoter is that of the HBV virus surface genes.

14. Vector according to claim 13, characterized in that it is the pHBV-S1.S2.S plasmid filed at the CNCM under N° I-1409.

15. Vector according to claim 7, characterized in that it includes an internal promoter.

16. Vector according to claim 6, characterized in that it comprises a cytoskeleton protein promoter.

17. Vector according to claim 16, characterized in that it comprises the desmine promoter.

18. Vector according to claim 16, characterized in that the promoter is homologous to the host to which the vector must be administered.

19. Vector according to claim 1, characterized in that it comprises the genes coding at least in part for the HIV1 virus gp160 protein associated to the p25 protein and/or the p55 protein and/or the p18 protein.

20. Vector according to claim 1, characterized in that it comprises at least one gene coding for the HIV1 virus Rev protein.

21. Nucleotide sequence comprising a promoter homologous to the host and another regulatory sequence for the expression of a gene or a DNA coding complement for S, S-preS$_2$ or S-preS1-preS2.

22. Nucleotide sequence comprising a promoter homologous to the host and another regulatory sequence for the expression of a gene or a complementary DNA coding for the gp160 protein associated with p25 and/or p55 and/or p18.

23. Nucleotide sequence comprising a promoter homologous to the host and another regulatory sequence for the expression of a gene or a DNA coding complement for the Rev protein.

24. Vaccine, characterized in that it contains at least one vector according to claim 1 or a nucleotide sequence according to claim 21.

25. Composition capable of inducing a cytotoxic response formed by at least one nucleotide sequence expressed in the muscle cells and including a promoter such as those defined in the claim 15.

26. Non-lipid pharmaceutical composition to be used in the immunization against a viral infection such as a hepatitis comprising of at least on the one hand a substance capable of inducing a coagulating necrosis of the muscle fibres and on the other hand a vector according to claim 1 or including the nucleotide sequence, complete or partial, according to claim 21.

27. Composition according to claim 26, characterized in that the substance is bupivacaine.

28. Composition according to claim 27, characterized in that the vector is administered into the muscle of the individual to be immunized, at least five days after the administration of bupivacaine, more or less in the same area.

29. Composition according to claim 28, characterized in that the vector is administered 10 days after administration of bupivacaine.

30: Composition according to one of the claims 26 to 29, characterized in that the administration is carried out by intramuscular injection.

31. Composition according to claim 30, characterized in that the intramuscular injection is carried out using a liquid jet gun.

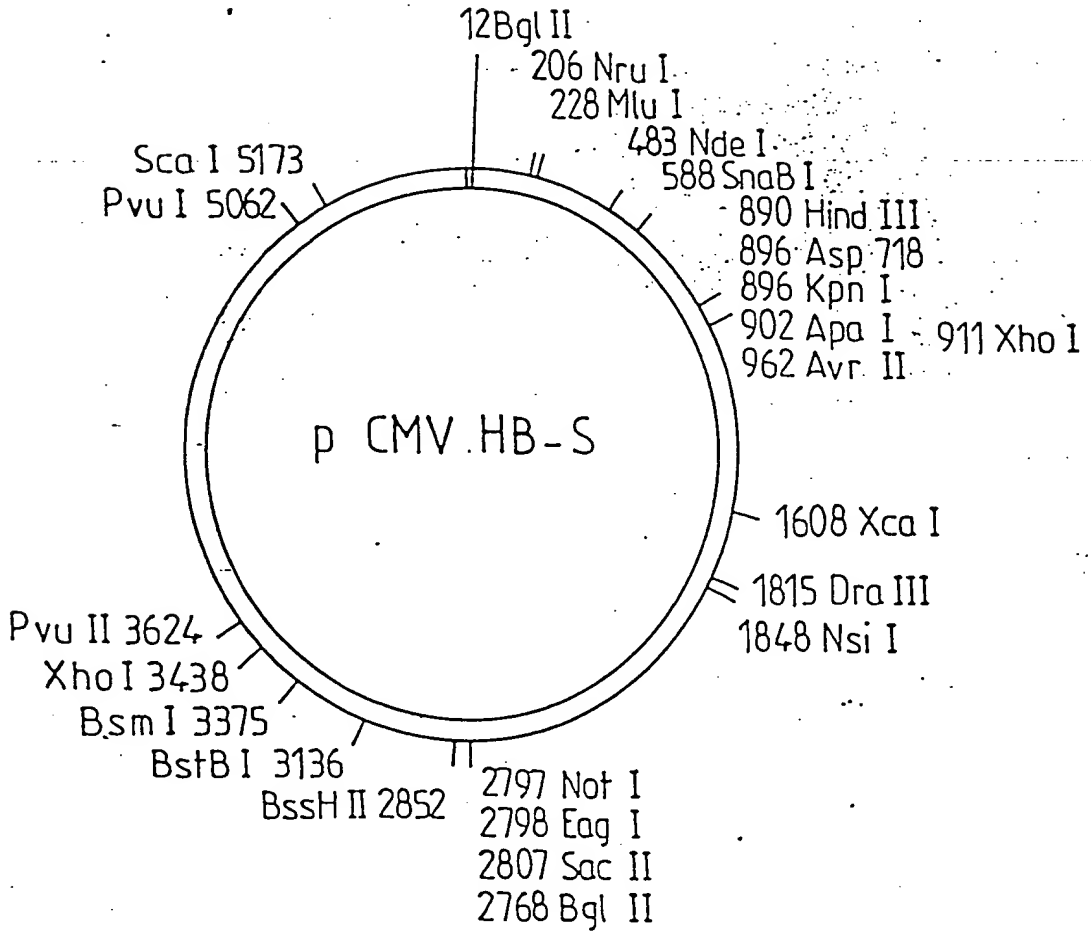
12Bgl II
206 Nru I
228 Mlu I
483 Nde I
588 SnaB I
890 Hind III
896 Asp 718
896 Kpn I
902 Apa I
911 Xho I
962 Avr II
p CMV HB-S
1608 Xca I
1815 Dra III
1848 Nsi I
2797 Not I
2798 Eag I
2807 Sac II
2768 Bgl II
BssH II 2852
BstB I 3136
Bsm I 3375
Xho I 3438
Pvu II 3624
Pvu I 5062
Sca I 5173

FIG.1

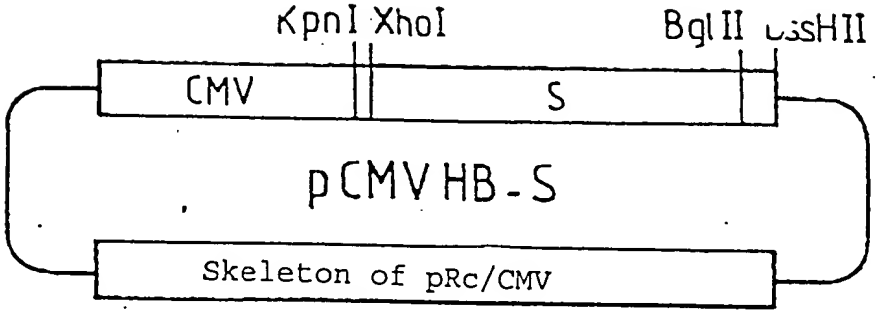

FIG.2A

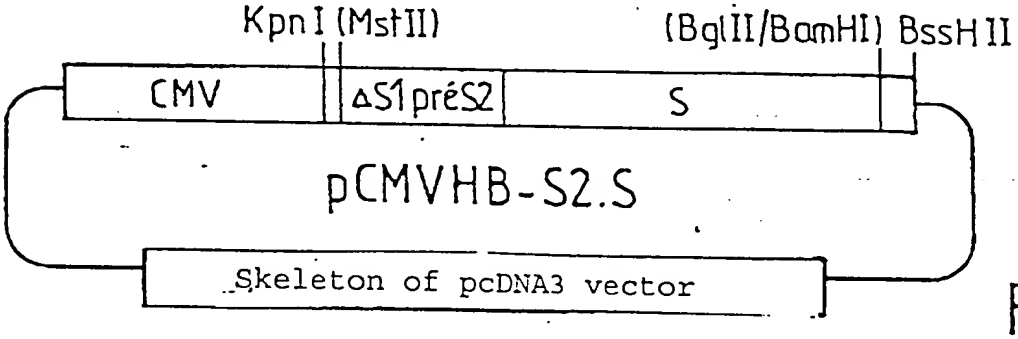

FIG.2B

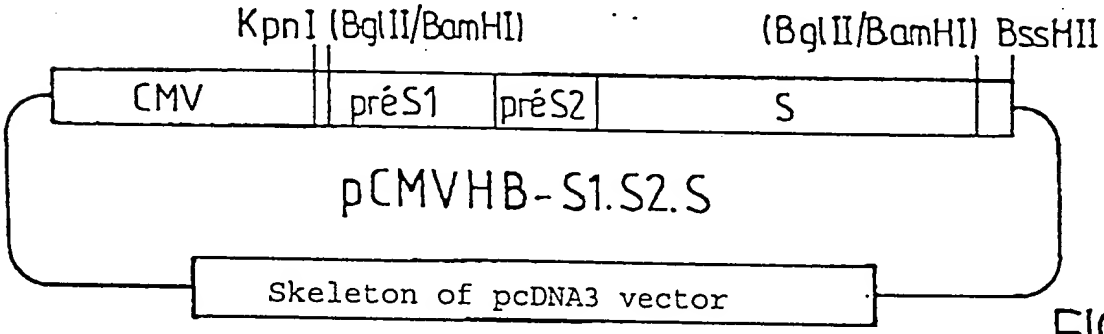

FIG.2C

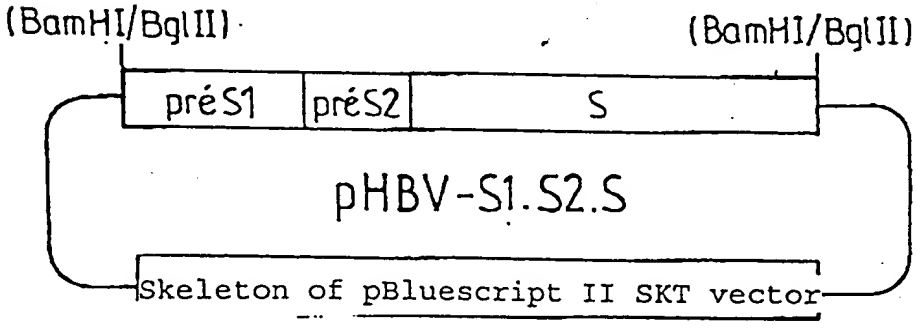

FIG.2D

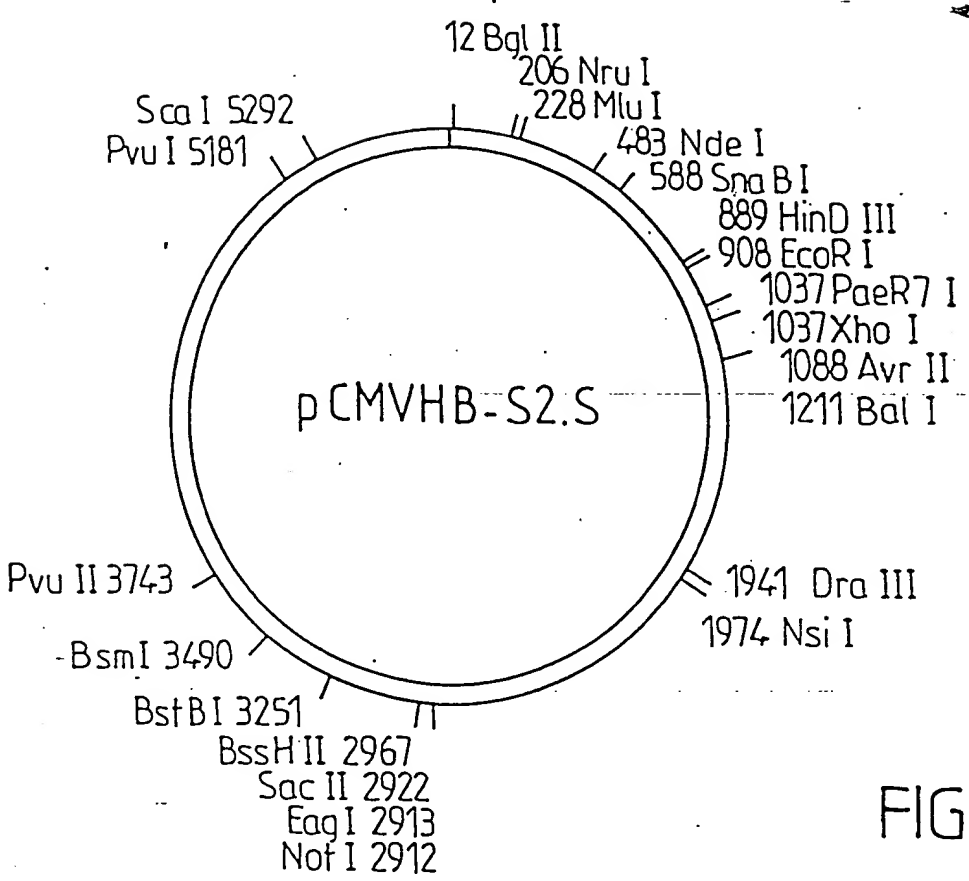
12 Bgl II
206 Nru I
228 Mlu I
483 Nde I
588 Sna B I
889 HinD III
908 EcoR I
1037 PaeR7 I
1037 Xho I
1088 Avr II
1211 Bal I
1941 Dra III
1974 Nsi I
Pvu II 3743
BsmI 3490
Bst B I 3251
BssH II 2967
Sac II 2922
Eag I 2913
Not I 2912
Sca I 5292
Pvu I 5181
pCMVHB-S2.S

FIG. 3

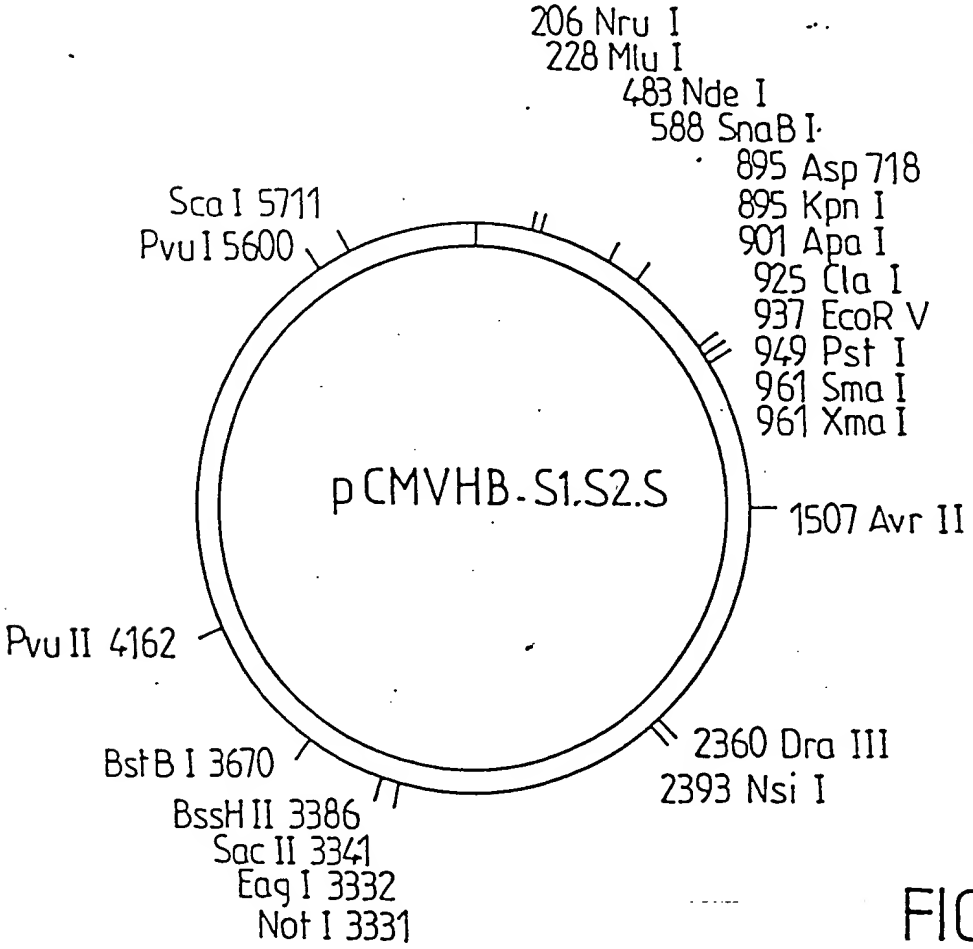
206 Nru I
228 Mlu I
483 Nde I
588 SnaB I
895 Asp 718
895 Kpn I
901 Apa I
925 Cla I
937 EcoR V
949 Pst I
961 Sma I
961 Xma I
1507 Avr II
2360 Dra III
2393 Nsi I
Pvu II 4162
Bst B I 3670
BssH II 3386
Sac II 3341
Eag I 3332
Not I 3331
Sca I 5711
Pvu I 5600
pCMVHB-S1.S2.S

FIG. 4

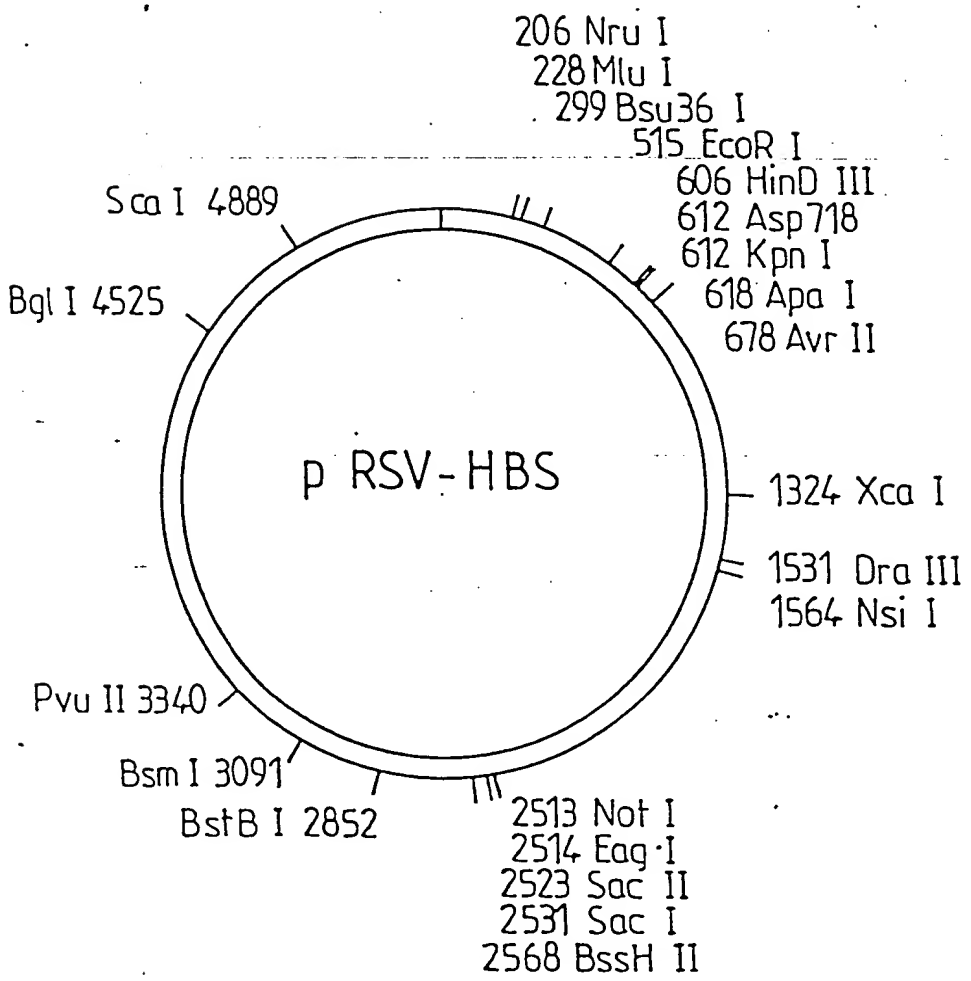
206 Nru I
228 Mlu I
299 Bsu36 I
515 EcoR I
606 HinD III
612 Asp718
612 Kpn I
618 Apa I
678 Avr II
Sca I 4889
Bgl I 4525
p RSV-HBS
1324 Xca I
1531 Dra III
1564 Nsi I
Pvu II 3340
Bsm I 3091
BstB I 2852
2513 Not I
2514 Eag I
2523 Sac II
2531 Sac I
2568 BssH II

FIG. 5

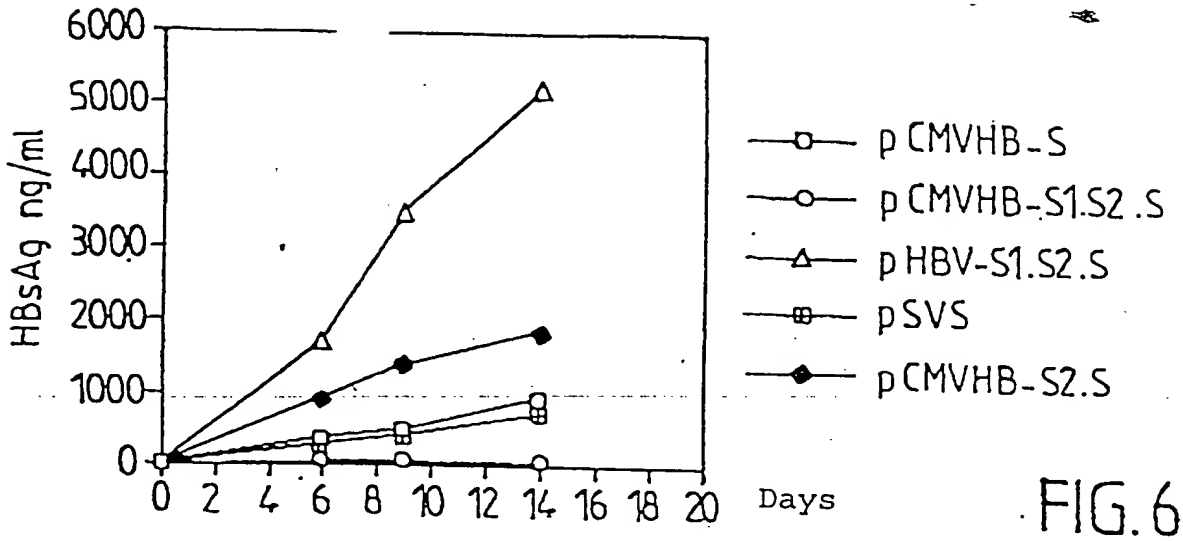

FIG. 6

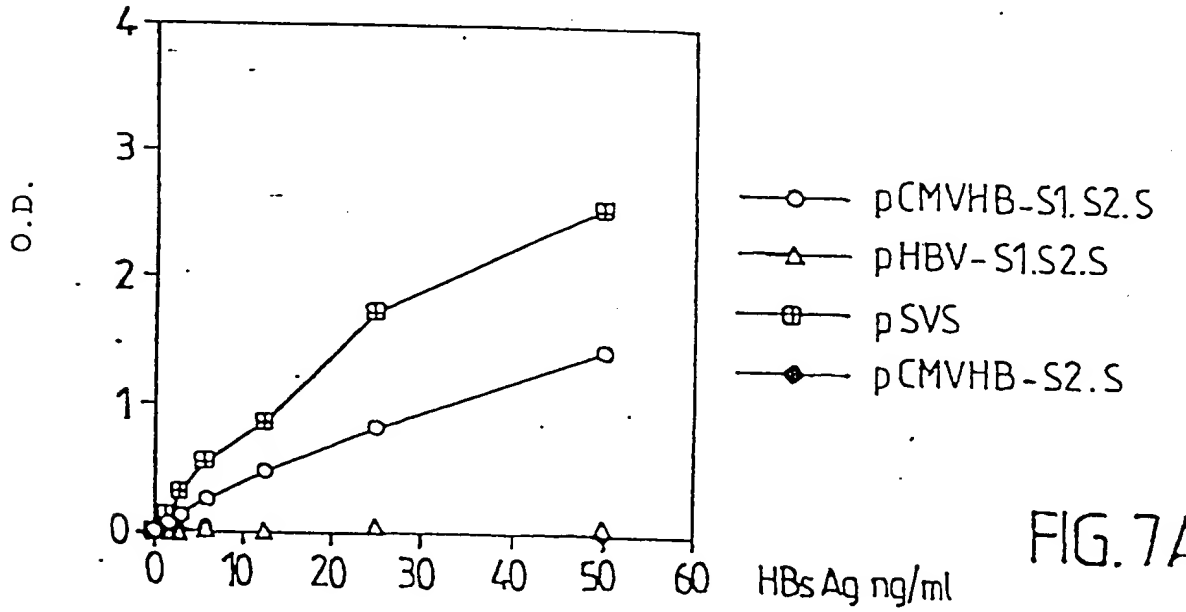

FIG. 7A

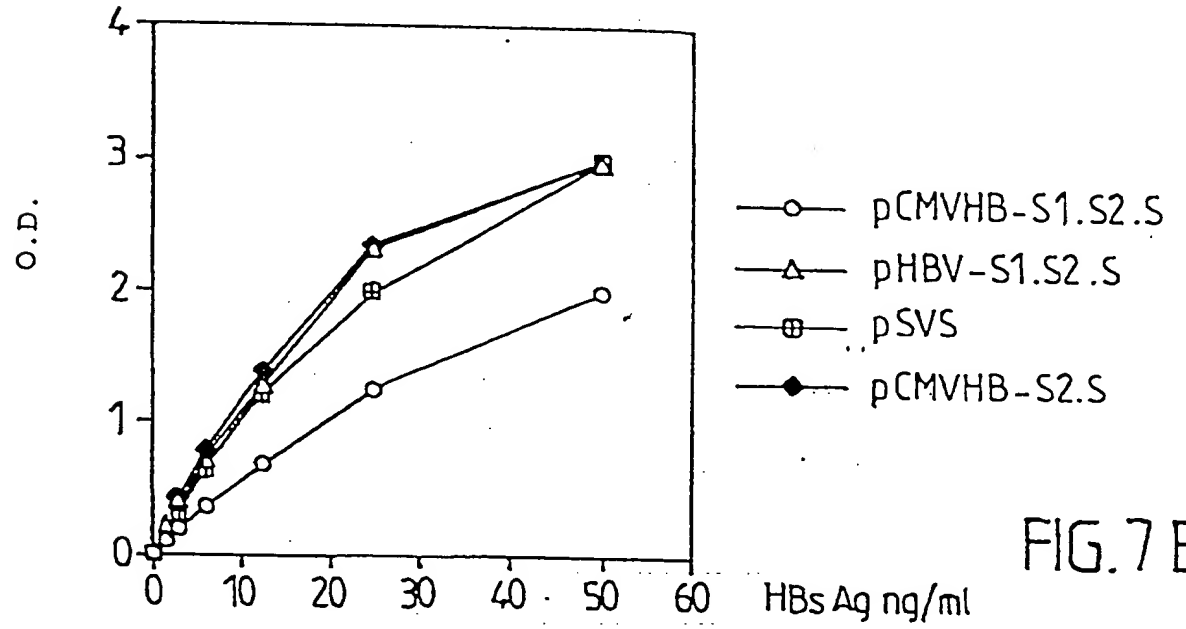

FIG. 7B

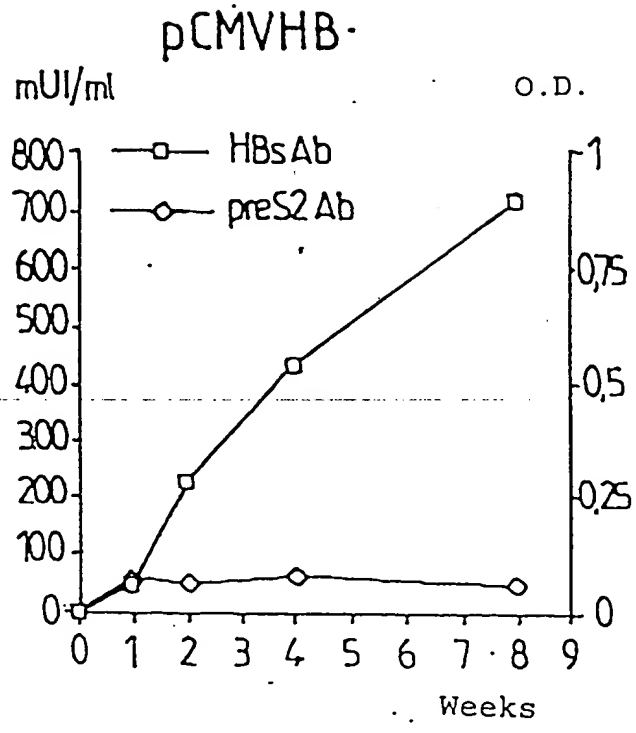

FIG.8A

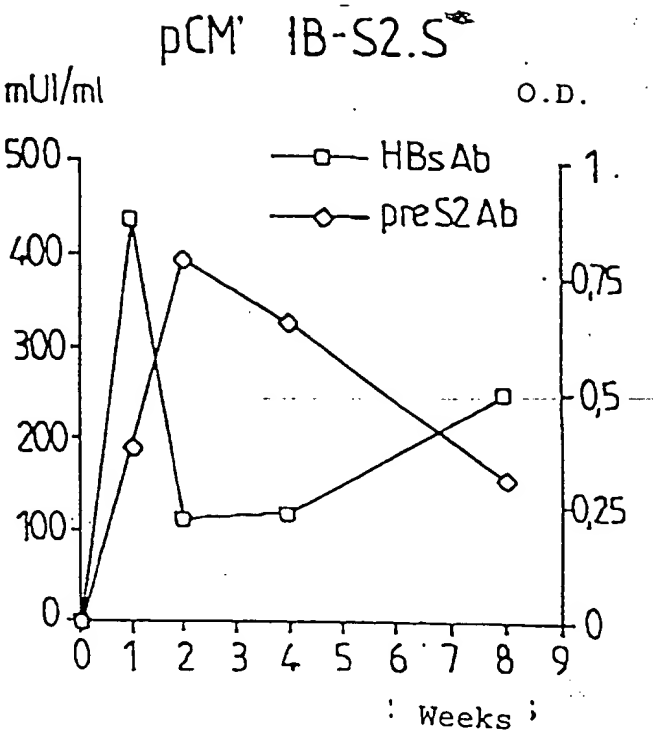

FIG.8B

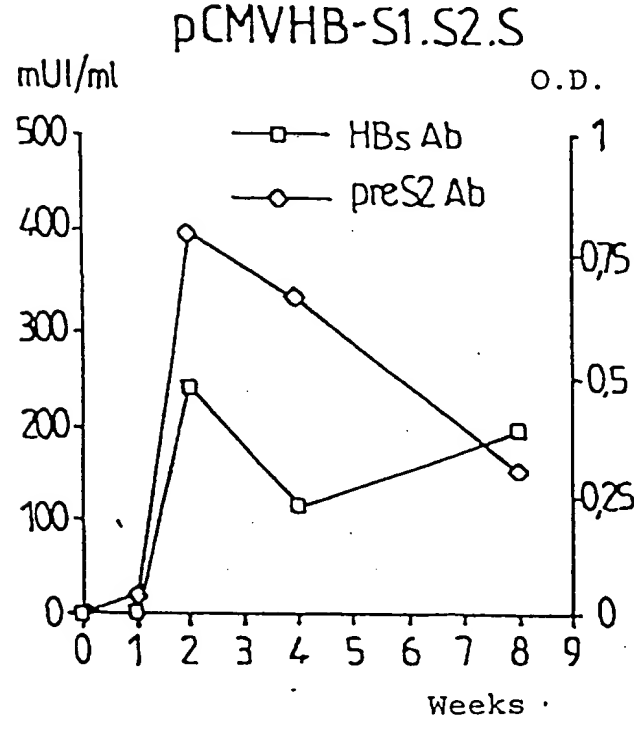

FIG.8C

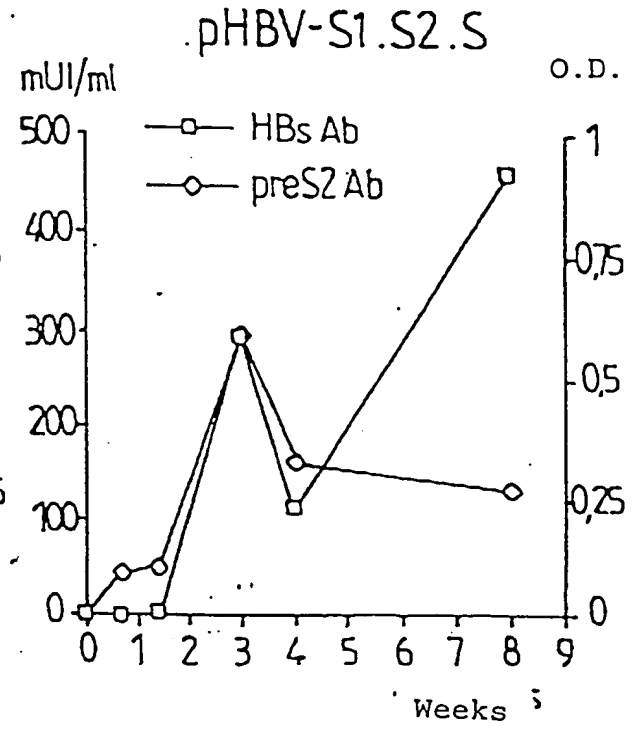

FIG.8D

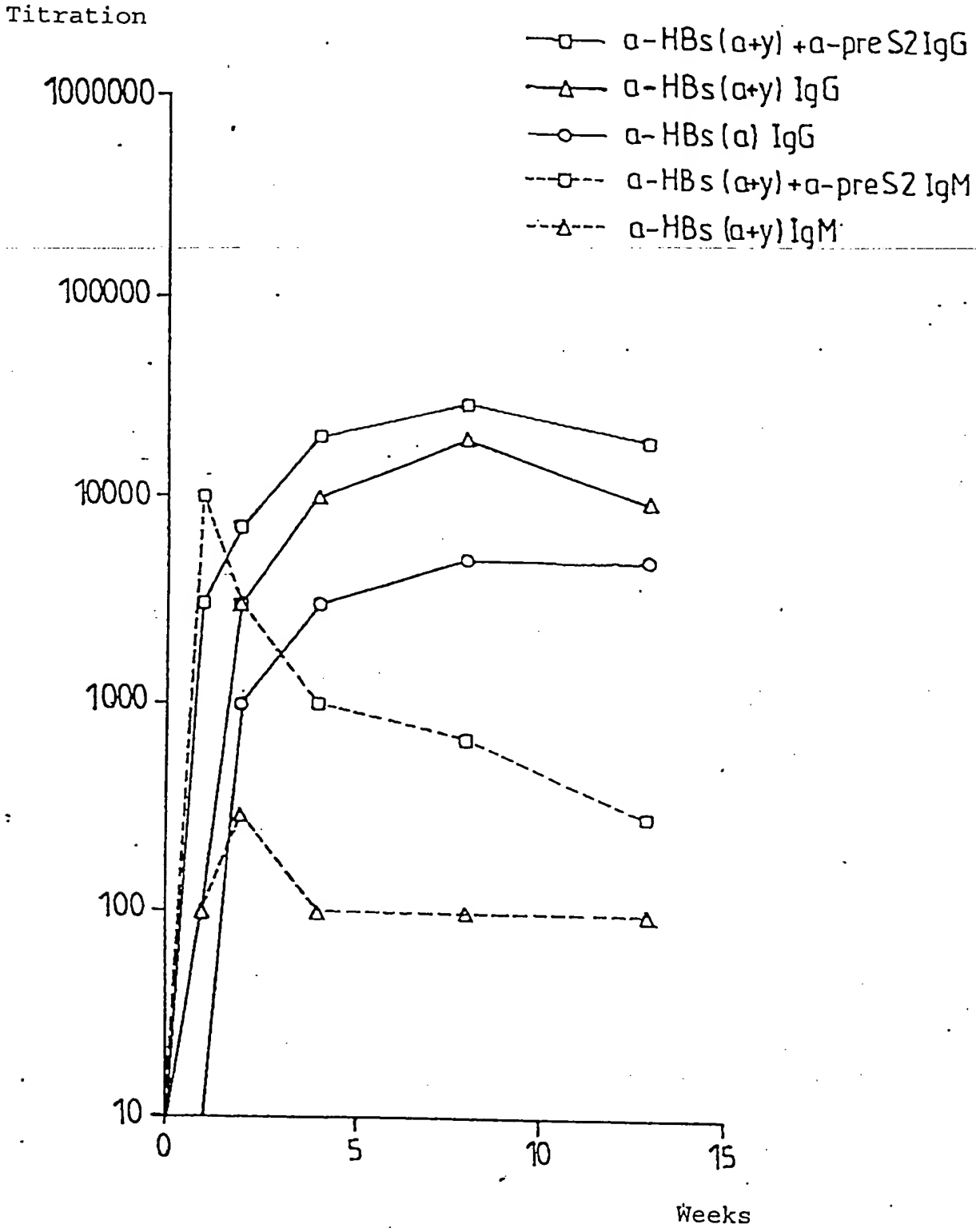
Titration
a-HBs(a+y)+a-preS2IgG
a-HBs(a+y) IgG
a-HBs(a) IgG
a-HBs(a+y)+a-preS2 IgM
a-HBs(a+y)IgM
1000000
100000
10000
1000
100
10
0
5
10
15
Weeks

FIG.9

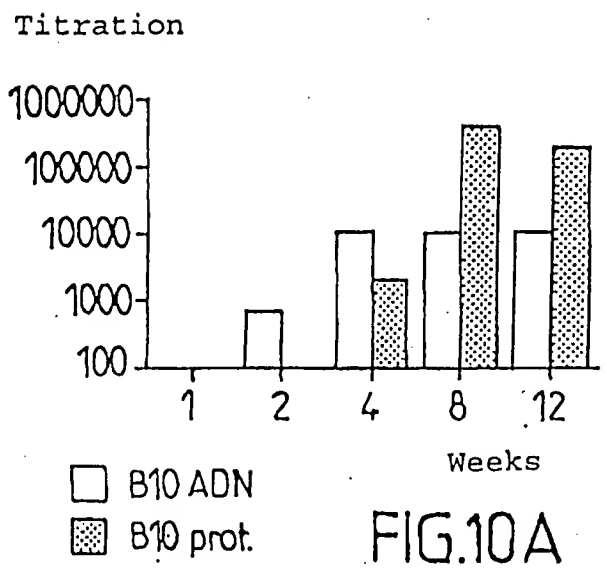

FIG.10A

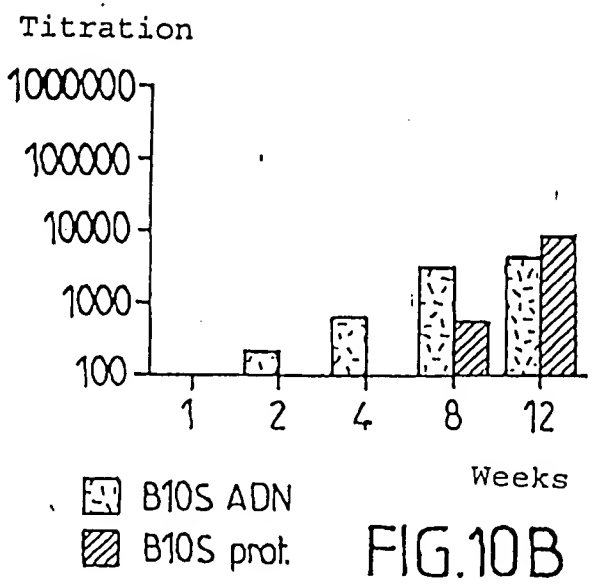

FIG.10B

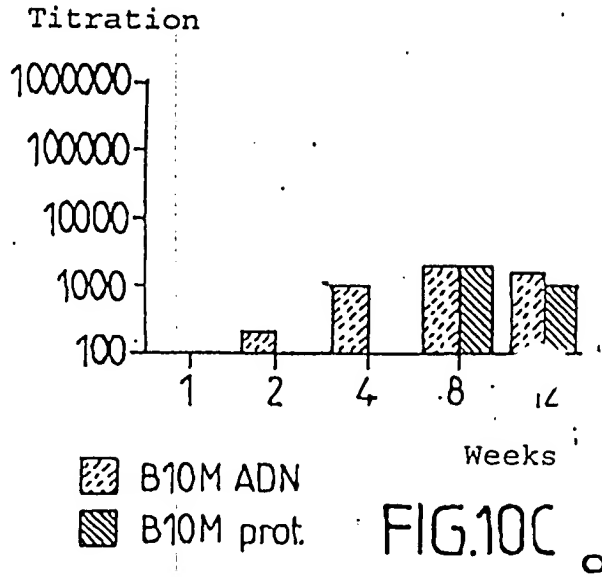

FIG.10C

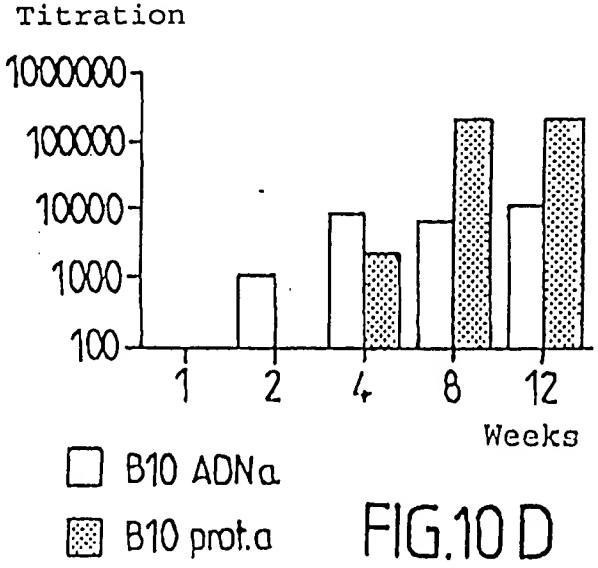

FIG.10D

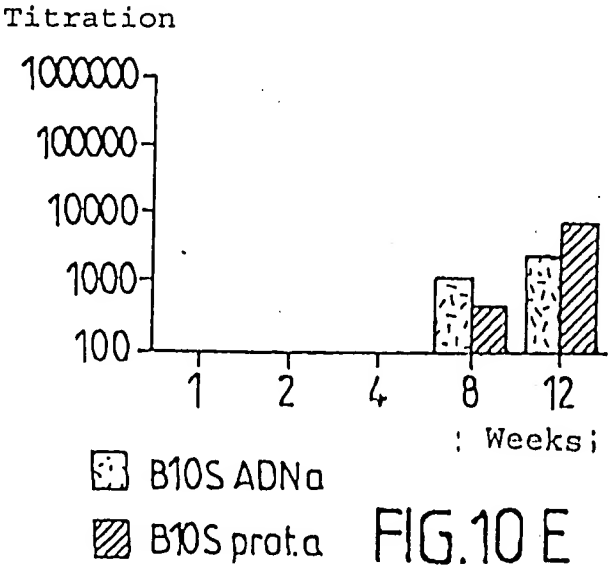

FIG.10E

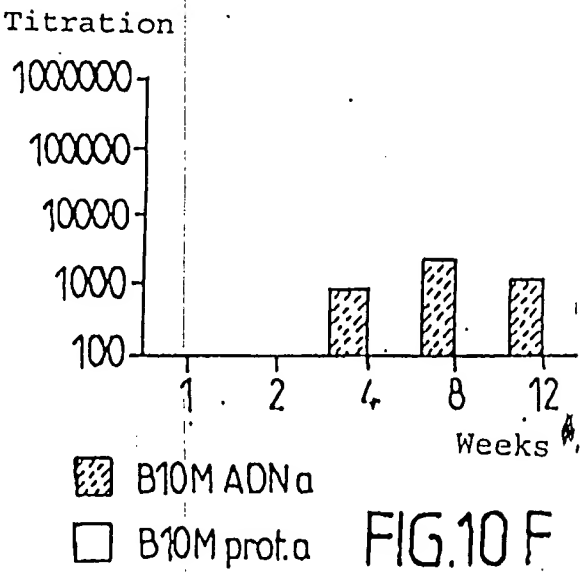

FIG.10F

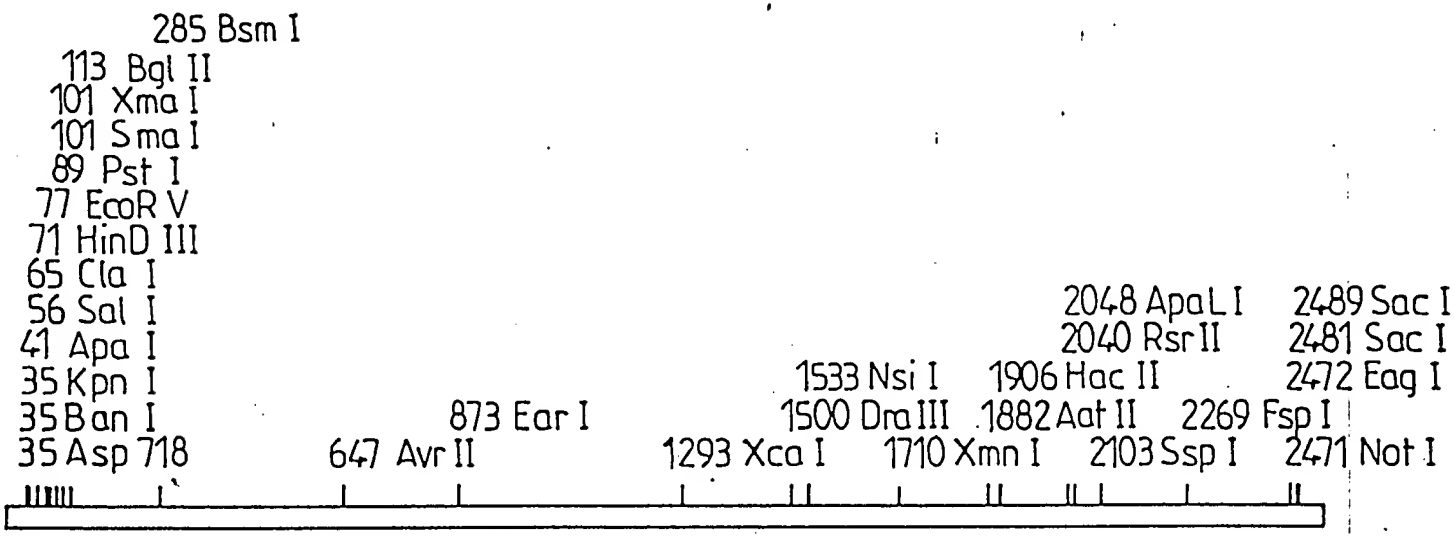
285 Bsm I
113 Bgl II
101 Xma I
101 Sma I
89 Pst I
77 EcoR V
71 HinD III
65 Cla I
56 Sal I
41 Apa I
35 Kpn I
35 Ban I
35 Asp 718
873 Ear I
647 Avr II
1533 Nsi I
1500 Dra III
1293 Xca I
1906 Hac II
1882 Aat II
1710 Xmn I
2048 ApaL I
2040 Rsr II
2269 Fsp I
2103 Ssp I
2489 Sac I
2481 Sac I
2472 Eag I
2471 Not I
pBS-SKT-S1.S2.S

FIG. 11